Meetings

Biological Membranes: Regulatory Functions

The second conference of the Sigrid Juselius Foundation on Regulatory Functions of Biological Membranes was held in Helsinki, Finland, 6–9 November 1967. The symposium was organized by Johan Jarnefelt. In his opening lecture, Sir Hans Krebs gave a most interesting and perceptive description of his researches on the effect of ethanol on the metabolism of the liver. He pointed out that ethanol inhibits gluconeogenesis from lactate. The primary effect is the lowering of the ratio of NAD to NADH in the liver cytoplasm, which in turn causes a rise in the ratio of lactate to pyruvate. The latter change involves a decrease in the steady-state concentration of pyruvate, one of the factors limiting the rate of gluconeogenesis from lactate and from various other precursors. The decrease in the ratio of NAD to NADH also causes an increase in the steady-state concentration of α-glycerophosphate, which is one of the factors controlling the synthesis of fat in the liver.

Correlations of the profiles of ethanol concentration with those profiles for the depression of gluconeogenesis by ethanol afforded the core of the experimental evidence for the control by alcohol dehydrogenase of gluconeogenesis and fat synthesis. However, in the discussion, it was pointed out that fatty livers do not show altered ratios of lactate to pyruvate at high ethanol levels. It was further noted that one explanation for the zero-order utilization of ethanol in wholebody experiments may well be the speed of the transmembrane shuttle for NADH from the cytoplasmic alcohol dehydrogenase to the mitochondrial oxidases. In one particular experiment, it was shown that the effects of ethanol leading to decreased gluconeogenesis need by no means be exclusively in the cytoplasmic space. In experiments with perfused rat liver, it was shown that addition of ethanol reduces flavoprotein of the mitochondrial space through the transmembrane hydrogen transfer pathway almost as fast as NAD is reduced in the cytoplasm by alcohol dehydrogenase.

Sjöstrand described extensive and carefully controlled electron micrographic studies of the membranes of intact mitochondria, amplifying his previous studies of the globular lipoproteins which comprise the membrane itself. Such units, of 50 to 60 angstroms in diameter, contain peripheral spots of 5 to 10 angstroms which stain clearly with uranyl acetate and outline the lipoproteins. Sjöstrand identifies these lipoproteins with the electron transfer components of the mitochondrial structure. Such components are placed in juxtaposition with one another, thereby obtaining a high relative concentration of both enzyme and substrate. He observes the formation of the projecting inner membrane subunits as extrusions from the mitochondrial membranes attendant to the formation of tubular extrusions from the crista, and in this sense, continues to cite the subunits as artifacts of preparation.

Other aspects of the structure of the membrane were taken up by Crane, who examined in detail fragments of the membrane obtained through treatment with amyl alcohol, deoxycholate, and Triton-X-114. String-like structures together with cytochromes b and c_1 were concluded to lie on a more substantial matrix containing cytochromes $a + a_3$. This reaction has led Crane to propose a parallel-plane structure for the respiratory chain, employing a base sheet of cytochromes $a + a_3$ upon which cytochromes b, c_1, and structural protein, as well as the inner membrane subunits, are situated. This structure, however, did not take into account the location of cytochrome c or of the dehydrogenases.

Three papers on membrane lipids by van Deenen, Kamat, and Wallach emphasized, respectively, precise studies on the chemical composition of the lipids, the NMR properties of the membranes, and their molecular architecture. Wallach attempted to correlate lipid and protein structure and proposed a model in which the hydrophobic portion of the protein helix is exposed at the exterior of the protein and closely interacting with the membrane lipids; the hydrophilic portion of the helix forms a channel through the membrane, presumably for transport processes. This structure was compared with the preliminary x-ray crystallographic structure of cytochrome c, the only membrane component of any system for which crystallographic data are available. Here, the hydrophobic space is on the inside rather than the outside. This configuration fits Sjöstrand's model which proposes that the lipids project into these hydrophobic spaces to anchor the protein to the membrane.

Metcalf described NMR measurements of T_2 for benzyl alcohol interacting with red cell membranes. He believed he could detect a point of liquefaction of the lipoprotein membrane for the added benzyl alcohol.

Lassen has measured the membrane potential and membrane resistance of red cells over short-time intervals. His apparatus consists of a microelectrode, 0.18 micron in diameter, and with a resistance of 10 to 20 megohms, driven piezo-electrically into the cell. Lassen finds the membrane potential to be -10 millivolts (maximum -14 millivolts) and the membrane resistance 4 megohms for an interval of 50 milliseconds. The result affords the first determination that the activity coefficient of the chloride ions inside the cell is the same as on the outside. A number of precautions to avoid contact potential errors were considered; the computed value for liquid junction potential was only $+1.3$ millivolts. It was apparent that the red blood cells were seriously damaged by their puncture by a needle of even these small dimensions, and it is possible that the membrane of the red cells differs from those of other types of cells and, therefore, does not adhere to the electrode tip satisfactorily.

Edelman, Leaf, and Rousseau presented three steps in the explanation of the regulation by aldosterone of sodium transport in the bladder of the toad. Edelman emphasized the role played by oxidative metabolism. Leaf attempted to distinguish between mitochondrial and cytoplasmic effects by various inhibitors and analytical tests. He ob-

served no stimulation of metabolism by aldosterone in the absence of external sodium, and concluded that aldosterone may create favorable conditions, perhaps inducing a permease, for bringing sodium into the tissue, thereby stimulating energy metabolism. These ideas were given some experimental support by Rousseau's tentative identification of two fractions of rapidly labeling ribonucleic acid, one believed to be a ribosomal RNA precursor and the other to be messenger RNA. The results, however, were preliminary. The exact correlation of the amounts of these fractions with the increase of sodium transport induced by aldosterone are not yet available. The discussion centered upon the difficulties of bioassays of intermediates in intact tissue. Leaf's general viewpoint was that the activity of the cells transporting ions was probably large compared with that of the other cells of the bladder membrane, and that the analytical results would, therefore, be meaningful.

Post's yields of the phosphorylated intermediate of Na^+ transport in the kidney membrane system are 8 $\mu\mu$mole of P^{32} per milligram of protein. The stability of the intermediate in the presence of varying amounts of sodium and potassium, as well as the profile for its *p*H sensitivity, now tentatively identify it as being of the acyl phosphate type.

Crofts indicated how the light-induced movements of hydrogen ions in thalakoid membranes from chloroplasts could be used to accumulate large amounts of ammonia and amines, particularly tetramethylene diamine, at relatively high initial rates. The efficiency of the process was found to reach three hydrogen ions per electron which, while high, is about half that reached in related reactions by other investigators in the field. In addition, Crofts demonstrated that oxidative phosphorylation can inhibit the uptake of ammonium ions, while at high concentrations of ammonium ions, the oxidative phosphorylation is inhibited by the uptake of ammonium ions.

In the discussion, it was pointed out that a primary hydrogen ion pump would require a compulsory time relationship between the movement of hydrogen ions and that of the electrons in the photosynthetic chain; the latter is known to be very rapid. Experiments on this point, employing rapid optical methods and appropriate *p*H indicators, have so far not revealed a close correlation between the oxidation-reduction steps and the speed of movements of hydrogen ions. Post pointed out that these membranes seemed highly permeable to hydrogen ions as evidenced by the rapid collapse of the light-induced concentration of ammonium ion gradients when the light is turned off. Thus, the thalakoid membranes represent a relatively inefficient system for energy conservation. In a discussion comment, Passow reported that ferricyanide external to the erythrocyte membrane can activate the ion pump in a reaction that is sensitive to oligomycin and to DNP. He proposed that an unknown electron-transporting system capable of ATP formation is located in the erythrocyte membrane.

Chemiosmosis was also discussed in some depth in an informal discussion. Data on the very rapid H^+ permeability into the matrix space containing the NAD-NADH system in the mitochondria of rat liver and pigeon heart were presented. The rate of entry of the hydrogen ions into the matrix so rapidly affects the ratio of NAD to NADH that the concept of a proton gradient across the mitochondrial membrane may be questioned. Ernster made the further point that the direction of proton movements in submitochondrial particles, being identical to that in chloroplasts and chromatophores, should lead to the uncoupling of the submitochondrial particles by ammonium ions, which are so effective in the latter two species. Since such effects are not observed, there is some question as to whether the proton gradient exists in these particles. Chance reported on the studies by Harris and Pressman on the use of nigericin to cause a massive potassium leak in mitochondria which did not, however, alter their oxidative phosphorylation properties. Alteration of the permeability properties of the membrane in this way would be expected to affect any hydrogen ion gradient or electrostatic potential across the membrane.

There was much discussion revolving about the variable properties of the mitochondrial membrane. Azzone stated that mitochondrial membranes washed with EDTA were permeable to Na, and K^+, and Ca^{++} as well, leading to the suggestion that magnesium may stabilize a structural element involved in the impermeability of the membrane. Crofts emphasized that small anions, such as acetate, are the only ones which do not seem to have a special permeability requirement.

Even this simplification was objected to by some, the general feeling being that special mechanisms were necessary for the transport of any species across the mitochondrial membrane. Some estimates of the speed of such processes were pointed to: in model experiments by M. Eigen, quoted by Hess, very fast changes of Rhodamine *b* were observed, while Chance noted slower changes in the reaction of bromthymol blue with the intact mitochondrial membrane. Azzi, using the rapid-flow apparatus in Chance's laboratory, finds that the initial binding of the indicator occurs in a time of 30 to 50 milliseconds, and is independent of the state of the mitochondria, as would be expected for binding to a structural element. Thus these indicators can respond very rapidly in the membrane system.

Saris put forward a chemical explanation for the observations made in several laboratories that bromthymol blue bound to mitochondria responds in the opposite direction from the glass electrode when hydrogen ions involved in electron transport move through the membrane. A careful study of the components of the mitochondria indicated binding of bromthymol blue to the "structural protein" fraction to be 10- to 40-fold that of the mitochondria themselves. The characteristic shift of the indicator *pK* from 7.2 to 8.2 observed in the mitochondria was reproduced with the indicator binding to the structural protein. The affinity of the structural protein for the indicator was found to be so high that no accurate value for the dissociation constant was obtainable. These results support other evidence that the structural protein is on the inside of the permeability barrier of the mitochondrial membrane and, in addition, afford a chemical explanation for the intramitochondrial *p*H indication obtained with bromthymol blue. Unfortunately, "structural protein" is at present a highly heterogeneous subfraction of mitochondrial protein.

Azzone reported some apparently anomalous responses of mitochondrially bound bromthymol blue to additions of inhibitors, substrates, and ATP which could be interpreted to show that the indicator was dissociable from its binding site. In the ensuing discussion, it was pointed out that the tight binding of the indicator to the structural protein suggested its dissociation velocity to be insufficient to explain the fairly rapid changes observed by Azzone. In addition, it was noted that it would be im-

possible to distinguish between movement of the indicator from one space to the other and a structural change in the mitochondria which rendered the structural protein accessible to a greater or lesser degree to the indicator.

Studies of the de-energized mitochondrial membrane were also reported by Azzone. He observed that after K^+ was added to mitochondria and then treated with Ca^{++}, the uptake of Ca^{++} and the efflux of K^+ occurred in a reaction that was abolished by uncouplers. Parallel experiments quantitated the Ca^{++} accumulation by isotopic procedures. The general conclusion was that the efflux of K^+ causes a potential across the membrane which is responsible for the uptake of Ca^{++}. In the discussion it was noted that energy-independent Ca^{++} uptake into mitochondria supplied with phosphate could be caused by the precipitation of calcium phosphate within the membrane. However, the experiments were also carried out in the absence of added phosphate. Since mitochondria contain endogenous phosphate, the question could be resolved by measuring the balance of ionic constituents, as emphasized by Edelman.

Ernster put forward a new explanation for the oxaloacetate inhibition of succinate oxidation in aged mitochondria. The evidence focused sharply on the possibility that a low concentration of free fatty acids present in the mitochondria or added to them served, in the presence of NAD or ATP, to activate electron transfer in the aged system. The remarkable feature of the experimental data was the large change of the inhibitor constant K_i for oxaloacetate before and after activation of respiration with fatty acids, ATP, and NAD. It was further pointed out that oxaloacetate had a remarkable affinity for the partly purified succinic dehydrogenase, suggesting that this bound oxaloacetate was involved in the deactivation phenomenon characteristic of the solubilized enzyme.

During the discussion, it was pointed out that a common denominator of the fatty acid activation of succinic dehydrogenase in aged mitochondria and succinate activation of the isolated succinic dehydrogenase could be a partial reduction of the succinic dehydrogenase flavoprotein prior to its reaction with succinate. This is a phenomenon related to the conditioning phenomenon of the NADH dehydrogenase of submitochondrial particles, where alteration in the availability of —SH groups

occurs after reduction with NADH. Smith reported an interesting phenomenon involved in propionate metabolism of mitochondria of sheep liver, where *dl*-carnitine showed considerable activating effects attributed generally to transport of the propionate across the mitochondrial membrane.

Papa described the pathway of α-oxoglutarate through the NAD-NADP system and glutamate dehydrogenase to form glutamate in mitochondria of rat liver. He postulated a separate compartment for the reaction of glutamate dehydrogenase and an energy requirement for the transfer of reducing equivalents to this site. The experimental results of Krebs pointed to an energy-dependent uptake of oxaloacetate. Energy supplied by Site III led to oxaloacetate uptake and NADH oxidation by the substrate. Papa showed that the rate of succinate oxidation in mitochondria of rabbit kidney is, in the presence of uncouplers, inversely correlated with the level of oxaloacetate. Hans Rasmussen showed how variable the response of the mitochondria to oxaloacetate might be. In one case, he was able to correlate an increase of succinate oxidation with an increase of intramitochondrial oxaloacetate in State 4 (mitochondria of pigeon heart supplemented with succinate).

Samuelsson described recent work on the prostaglandins and postulated interesting and fundamental interactions with the cyclic AMP and lipase.

Higgins reviewed factors contributing to the instability of enzymatic systems and emphasized, particularly, the back-activation oscillator as a representative example. He further illustrated a phenomenological comparison of oscillations in metabolites in an extract from a heart cell with analog computer solutions obtained by A. Lucas. He showed particularly how the entrainment of oscillations from the phosphofructokinase step to the 1,3-phosphoglyceric acid step could be identified in characteristic portions of the oscillation cycle. It was brought out in the discussion that manifold interactions are possible in the glycolytic sequence and the idea of a specific control site or rate-limiting step as controlling the properties of the whole sequence must be abandoned in favor of an understanding of the interaction of several controls.

Hess described a reconstruction of the oscillatory system in cell-free extracts, in which purified enzymes from hexokinase to alcohol dehydrogenase

were employed to obtain oscillations of unusual period and waveform. It was pointed out that one of the problems of employing purified enzymes for this purpose was that some of the isoenzymes might be missing, as in the case of glyceraldehyde-3-phosphate dehydrogenase.

Changeux reviewed the basis for the allosteric hypothesis and showed a molecular model of the aspartate transcarbamylase illustrating type of contact interactions between the subunits of the polymer. He then described a number of experiments on the electroplaques of the electric fish and pointed out how the allosteric hypothesis could be enlarged so as to predict some properties of the excitable membrane. There was some discussion as to whether the speed of subunit reorganization would be rapid enough for the great speed of propagated action potentials in small axons. It was also pointed out that propagation of information in a membrane system might well occur through changes in the secondary or tertiary structure affecting intermolecular contacts of protein molecules.

Cohn described in detail various types of nuclear magnetic relaxation data that could be obtained from a variety of metal-activated enzymes, such as creatine kinase and pyruvate kinase, where, in certain cases, the atomic dimensions of the metal-substrate distance for the enzyme-metal-substrate complex could be computed for the complex in solution. She further pointed to proton magnetic relaxation studies as an approach to manganese binding to the mitochondrial membrane. Enhancement of the proton relaxation rate due to manganese was observed when manganese was bound to the membrane. Other possible applications of this important method to metal-adenine nucleotide binding were pointed out.

Clark described an interesting fish poison named cunaniols (polyacetylenic alcohol) which appeared to have slow but highly significant effects upon the respiratory control of mitochondria of rat liver. Although the mitochondria provided a simple assay system for the cunaniols, the physiological target might well be in the central nervous system or in the lungs.

M. Baltscheffsky summarized her work on inorganic pyrophosphate as an energy donor, activating reversed electron transfer in chromatophores, in rat liver, and in yeast mitochondria. Particularly striking results were obtained in chromatophores from the bluegreen mutant of *Rhodospirillum rubrum*. This finding evoked discussion; a summary was made of previous, unsuccessful attempts by Lindberg to demonstrate by isotopic methods the labeling of the adenine nucleotide pool of mitochondria by labeled pyrophosphate. It is apparent that reversed electron transfer is a highly sensitive and specific reaction for energy donors and thus affords a higher ratio of signal to background than could be obtained with other techniques. The possible role of this reaction in biochemical evolution was stressed by H. Baltscheffsky.

Vernon reported on the isolation of subchloroplast and subchromatophore fractions in which the various activities of the system could be segregated to a remarkable degree. His general conclusion was that the System I particle, consisting of rods or strings, contains cytochromes *f* and b_6, while the vesicular System II particle consisting of vesicles contains a cytochrome b_{559}. Recombination of these fractions to make the complete unit of System I–System II has not yet been reported. Comparable studies of *Chromatium* chromatophores revealed P_{890} and ubiquinone in the particles with no activity in the vesicles.

Jarnefelt described preliminary attempts to find a more appropriate medium for studying the metabolism and function of slices of brain cortex, with the ultimate goal of obtaining preparations which would exhibit spontaneous activity. Tata described elegant studies on hormonally induced membrane synthesis in a variety of organs, finding a very close coincidence in the biosynthesis of the membranes of the endoplasmic reticulum and of the mitochondria. These studies suggest that the bulk of the membranes of the endoplasmic reticulum and ribosomes are turned over as a unit, and that there is a good correlation between hormonal specificity in regulating the overall biosynthetic process.

The symposium was closed with a brief summary and a vote of thanks to M. von Knorring and P. Olin of the Sigrid Juselius Foundation; to Johan Jarnefelt for organizing the meeting; and to Sir Hans Krebs for his fine contribution to various phases of the proceedings.

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